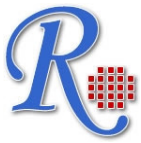
Quantibody® Mouse Ig Isotyping Array 1

--Quantitative measurement of 7 Mouse Immnunoglobulins

Patent Pending Technology

User Manual (Version 1/1/2015)

Cat # QAM-ISO-1



RayBiotech, Inc.

We Provide You With Excellent
Protein Array Systems and Service

Tel:(Toll Free) 1-888-494-8555 or 770-729-2992; Fax: 1-888-547-0580;
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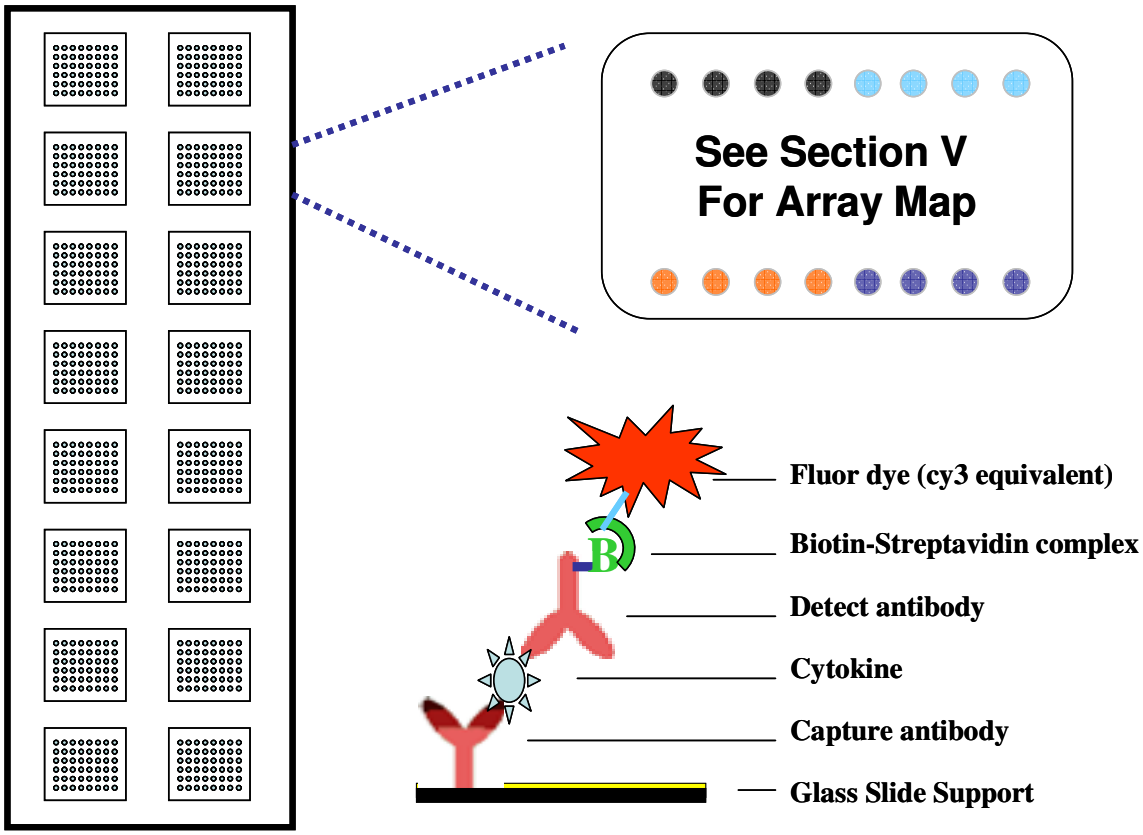
Immunoglobulin Detected (7+3)	Quantitative: IgA, IgE, IgM, IgG1, IgG2a, IgG2b, and IgG3 Semi-Quantitative: IgD and two light chains (κ, and λ)
Format	One standard glass slide is spotted with 16 wells of identical Immunoglobulin antibody arrays. Each antibody is arrayed in quadruplicate.
Detection Method	Fluorescence with laser scanner: Cy3 equivalent dye
Sample Volume	50 – 100 µl per array
Sample Dilution	Hybridoma Supernatant: 1:10 – 1:1,000 Purified mouse monoclonal antibody: 10-1000 ng/ml Ascitic fluids/Serum/Plasma: 1:1,000 – 1:100,000
Reproducibility	CV <20%
Assay duration	6 hrs



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I. Introduction

Immunoglobulins are the key elements of the humoral immune response in vertebrate against parasitic invasion. The polypeptide chains of immunoglobulins are composed of two identical heavy (H) chains and two identical light (L) chains linked together by inter-chain disulfide bonds. While the amino terminal portions that exhibits highly variable amino acid composition are involved in antigen binding, the C terminal constant parts are involved in complement binding, placental passage and binding to cell membranes. Based upon the variation of the constant region of the heavy chain, eight immunoglobulin heavy chain isotypes are found in mice: IgA, IgD, IgE, IgM, and IgG (with subclasses IgG1, IgG2a, IgG2b, and IgG3).

Identification of class and subclass of immunoglobulins is essential for determination of immunochemical and functional properties. Detection of specific Ig isotype is a powerful tool in the study of immunoglobulin-deficiency disorders, allergies, autoimmune diseases, malignancies, GI disorders or repeated bacterial infections. Meanwhile, the growth and widespread use of mouse monoclonal antibody technology have created a need for a fast, accurate, and simple means of determining immunoglobulin class and sub-class. Identification is essential since chemical and biological properties of the various classes are unique. They differ in their solubility and electrophoretic properties, in their susceptibility to cleavage enzymes, and in their reactivity with protein A. Determining the class and subclass of a monoclonal antibody is thus useful in planning the best immunoglobulin purification method. For example, mouse IgA and IgM are best purified by size (i.e., gel exclusion) or using immunoaffinity separation columns. Mouse IgG2a and IgG2b are purified with immobilized Protein A at pH 7-8, while Mouse IgG1 binds best to Protein A at pH 8-9. Immunoglobulin that contains kappa light chains can be purified using immobilized Protein L.

Quantitative measurement of the immunoglobulin subclasses can be done with Radial Immunodiffusion assay (RID), Nephelometry and turbidimetry assay, Radio Immuno Assay (RIA), Immuno-affnity chromatography, Direct Antiglobulin Test (DAT), or Enzyme-linked Immunosorbent Assay (ELISA). While most assays can detect only one subclass of the immunoglobulin a time, taking advantage of the array technology and the availability of the

isotype specific monoclonal antibodies, Raybiotech Inc is proudly offering the research community with the Quantibody® Mouse Ig Isotype kit which can simultaneously and quantitatively detect multiple immunoglobulin subclasses in one experiment.

Quantibody® Mouse Ig Isotype Array uses sandwich-ELISA based technology for quantitative measurement of the seven mouse isotype immunoglobulins (IgG1, IgG2a, IgG2b, IgG3, IgA, IgE, and IgM) and semi-quantitative measurement of IgD and the two light chain types. Similar technology has been successfully used in our other quantibody® products for quantitative measurement of up to 40 cytokines in human, mouse, rat, and porcine samples. (See Section XI). Briefly, the 7 mouse immunoglobulin subclass-specific antibodies are arrayed in quadruplicate (together with two positive controls) with 16 identical sub-arrays in one standard glass slide. The kit also provides a myeloma-derived standard mixture of these 7 immunoglobulins, whose concentration has been predetermined.

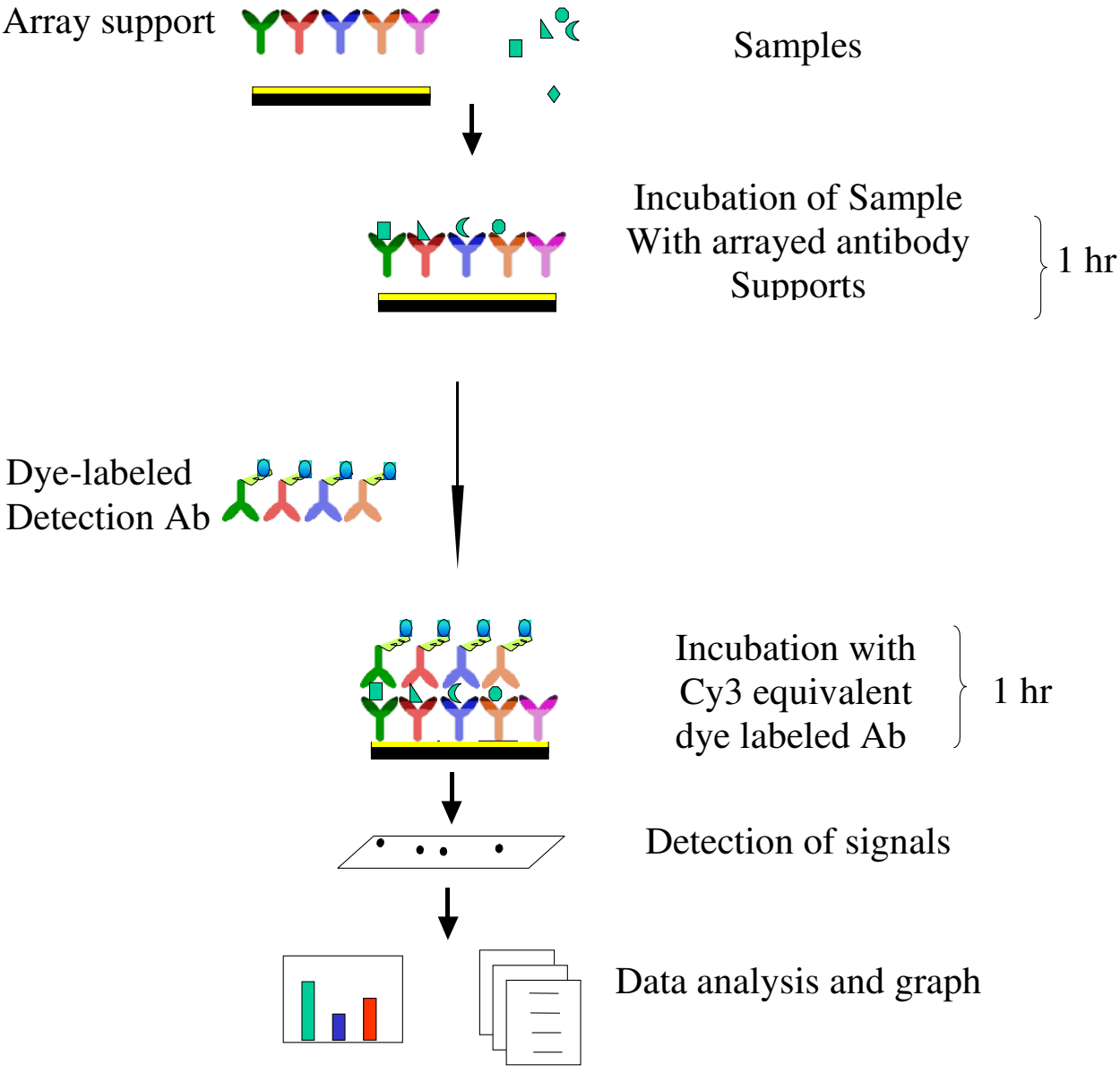
In the experiment, standard immunoglobulins and samples are assayed in each well simultaneously through a sandwich like ELISA procedure. The signals will be detected using fluorescence-based detection method for consistency and reliability. By comparing signals from unknown samples to the standard curve generated for each of the 7 immunoglobulins, the unknown immunoglobulin concentration in the samples will be determined.

The kit provides a highly sensitive approach (within nano gram range) to simultaneously detect 7 immunoglobulin subclasses expression levels. The experimental procedure is simple and can be performed in any laboratory. This kit can be used for many applications such as: Antibody-producing hybridoma screening and selection; Mouse monoclonal antibody heavy and light chain identification; Selection and isolation of immunoglobulin isotype switch variants; Mouse model immune disease research (autoimmune disease, allergies, Ig deficiency disorders, malignancies, GI disorders or repeated bacterial infections etc.).

For researchers who don't need quantitative data but only want to determine the mouse immunoglobulin isotypes, a semi-quantitative version is available: **Raybio® Rapid Mouse Ig Isotyping Array** (Cat# **AAM-ISO-1**). This semi-quantitative kit has following features:

- One step mouse monoclonal antibody isotyping
- Use only 1-2 µl sample
- The whole experiment can be done within 1 hour
- Sandwich based technology for high specificity and sensitivity
- Low system CV with high reproducibility
- High throughput sample processing
- Qualitative visual inspection or semi-quantitative result
- Processed slides can be stored for years without signal decay

How It Works


Array support
Samples
Incubation of Sample
With arrayed antibody
Supports
1 hr
Dye-labeled
Detection Ab
Incubation with
Cy3 equivalent
dye labeled Ab
1 hr
Detection of signals
Data analysis and graph

I. Materials Provided

Upon receipt, all components of the Quantibody® Array kit should be stored at -20^{0}C. At -20^{0}C the kit will retain complete activity for up to 6 months. Once thawed, the glass chip, Immunoglobulin standard mix, and detection antibody cocktail should be kept at –20^{0}C and all other components may be stored at 4^{0}C. The entire kit should be used within 6 months of purchase.

Components

Item	Description	1-Slide kit	2-Slide kit
1	Quantibody® Array Glass Chip	1	2
2	Sample Diluent	1	1
3	20X Wash Buffer I	2	3
4	20X Wash Buffer II	1	1
5	Lyophilized Immunoglobulin standard mix *	1	1
6	Dye labeled detection antibody	1	2
7	Slide Washer/Dryer	1	1
8	Adhesive device sealer	5	10
9	Manual	1	1

** See Section VI for detailed Immunoglobulin concentrations after reconstitution.*

Additional Materials Required

- Orbital shaker
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water
- 1.5ml Polypropylene microcentrifuge tubes

II. General Considerations

A. Preparation of Samples

- **Sample types:** hybridoma supernatant, purified antibodies, serum, plasma, and ascitic fluids etc.
- **Suggested sample dilution**:
  a) Hybridoma supernatant: 1:10 – 1:1,000
  b) Purified mouse monoclonal antibody: 10 – 1000 ng/ml
  c) Serum, plasma, and ascitic fluids: 1:1,000 – 1:100,000

B. Handling glass chips

- Do not touch the surface of the slides, as the microarray slides are very sensitive. Hold the slides by the edges only.
- Handle all buffers and slides with latex free gloves.
- Handle glass chip in clean environment.
- Because there is no barcode on the slide, transcribe the slide serial number from the slide bag to the back of the slide with a permanent marker before discarding the slide bag. Once the slide is disassembled, you might not have enough info to distinguish one slide from the other.

C. Incubation

- Completely cover array area with sample or buffer during incubation.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.
- Cover the incubation chamber with adhesive film during incubation, particularly when incubation is more than 2 hours or <70 µl of sample or reagent is used.
- Several incubation steps such as step 6 (blocking), step 7 (sample incubation), or step 10 (detection antibody incubation) may be done overnight at 4^0C. Please make sure to cover the incubation chamber tightly to prevent evaporation.

III. Protocol

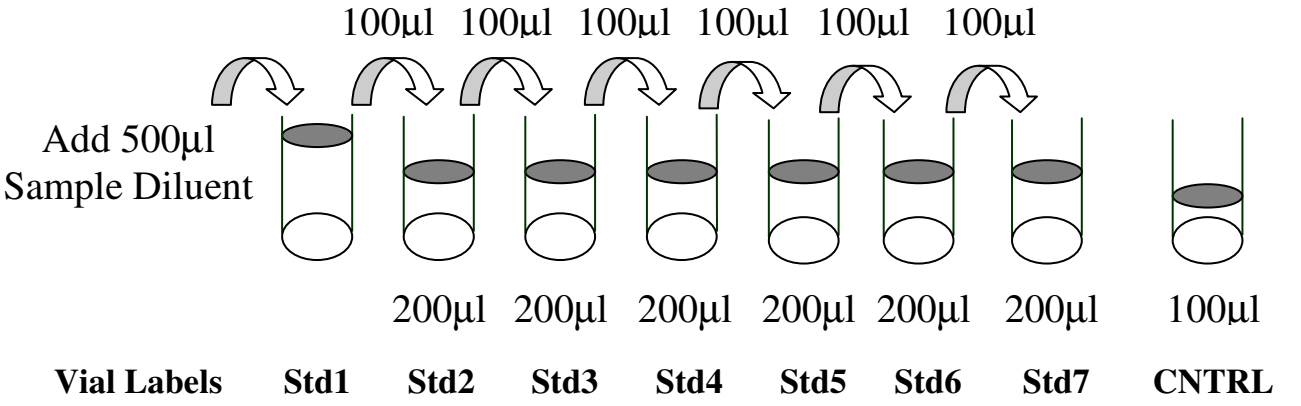
A. Completely air dry the glass chip

1. Take out the glass chip from the box, and let it equilibrate to room temperature inside the sealed plastic bag for 20-30 minutes. Remove slide from the plastic bag; peel off the cover film, and let it air dry at room temperature for another 1-2 hours.

Note: Incomplete drying of slides before use may cause the formation of "comet tails".

B. Prepare Immunoglobulin Standard Dilutions

Prepare serial dilution of Immunoglobulin standards


100µl 100µl 100µl 100µl 100µl 100µl
Add 500µl
Sample Diluent
200µl 200µl 200µl 200µl 200µl 200µl 100µl
Vial Labels Std1 Std2 Std3 Std4 Std5 Std6 Std7 CNTRL

2. Reconstitute the Immunoglobulin Standard Mix (lyophilized) by adding 500µl Sample Diluent to the tube. For best recovery, always quick-spin vial prior to opening. Dissolve the powder thoroughly by a gentle mix. Labeled the tube as Std1.
3. Label 6 clean microcentrifuge tubes as Std2 to Std7. Add 200µl Sample Diluent to each of the tubes.
4. Pipette 100µl Std1 into tube Std2 and mix gently. Perform 5 more serial dilutions by adding 100ul Std2 to tube Std3 and so on.

5. Add 100µl Sample Diluent to another tube labeled as CNTRL. Do not add standard Immunoglobulin or samples to the CNTRL tube, which will be used as negative control. For best results, include a set of standards in each slide.

Note: Since the starting concentration of each Immunoglobulin is different, their serial concentrations from Std1 to Std7 are varied which can be found in section VI.

C. Blocking and Incubation

6. Add 100µl Sample Diluent into each well and incubate at room temperature for 30 min to block slides.

7. Decant buffer from each well. Add 100µl standard Immunoglobulins or samples to each well. Incubate arrays at room temperature for 1 hour. *(Longer incubation time is preferable for higher signals)*

   Note: The sample volume can be 50-100 µl. If sample volume is less than 70 µl, cover the gasket with adhesive sealer to prevent evaporation during incubation. This step may be done overnight at 4^0C for best results.
   Note: See Section II for recommendations of sample dilutions.

8. Wash:
   - Decant the samples from each well, and wash 5 times (5 min each) with 150 µl of 1x Wash Buffer I at room temperature with gentle shaking. Completely remove wash buffer in each wash step. Dilute 20x Wash Buffer I with H_2O.
   - (*Optional*) Put the glass chip with frame into a box with 1x Wash Buffer I (cover the whole glass slide and frame with Wash Buffer I), and wash at room temperature with gentle shaking for 20 min.
   - Decant the 1x Wash Buffer I from each well, wash 2 times (5 min each) with 150 µl of 1x Wash Buffer II at room temperature with

gentle shaking. Completely remove wash buffer in each wash step. Dilute 20x Wash Buffer II with H_2O.

Note: Incomplete removal of the wash buffer in each wash step may cause "dark spots". (Background signal is higher than that of the spot.)

D. Incubation with dye labeled detection antibody and wash.

9. Reconstitute the detection antibody by adding 1.4 ml of Sample Diluent to the tube. Spin briefly.

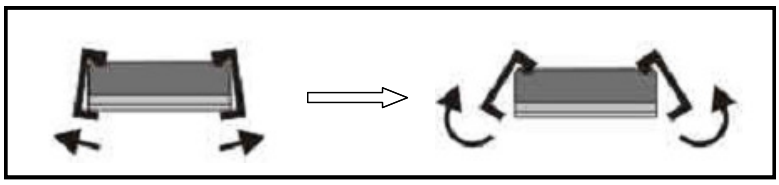
10. Add 80 µl of the dye labeled detection antibody to each well. Incubate at room temperature for 1 hour. *(Longer incubation time is preferable for higher signals and backgrounds)*

11. Decant the samples from each well, and wash 5 times with 150 µl of 1x Wash Buffer I and then 2 times with 150 µl of 1x Wash Buffer II at room temperature with gentle shaking. Completely remove wash buffer in each wash step.

E. Fluorescence Detection

12. Disassemble the device by pushing clips outward from the slide side. Carefully remove the slide from the gasket.

(Be careful not to touch the surface of the array side)



13. Place the slide in the slide Washer/Dryer (a 4-slide holder/centrifuge tube), add enough 1x Wash Buffer I (about 30 ml) to cover the whole slide, and then gently shake at room temperature for 15 minutes. Decant Wash Buffer I. Wash with 1x Wash Buffer II (about 30 ml) with gentle, and gently shake at room temperature for 5 minutes.

14. Remove water droplets completely by gently applying suction with a pipette to remove water droplets. Do not touch the array, only the sides.

    Note: You may dry the glass chip by a compressed N_2 stream.

15. Imaging: The signals can be visualized through use of a laser scanner equipped with a Cy3 wavelength such as Axon GenePix. Make sure that the signal from the well containing the highest standard concentration (Std1) receives the highest possible reading, yet remains unsaturated.

Note: In case the signal intensity for different Immunoglobulin varies greatly in the same array, we recommend using multiple scans, with a higher PMT for low signal Immunoglobulins, and a low PMT for high signal Immunoglobulins.

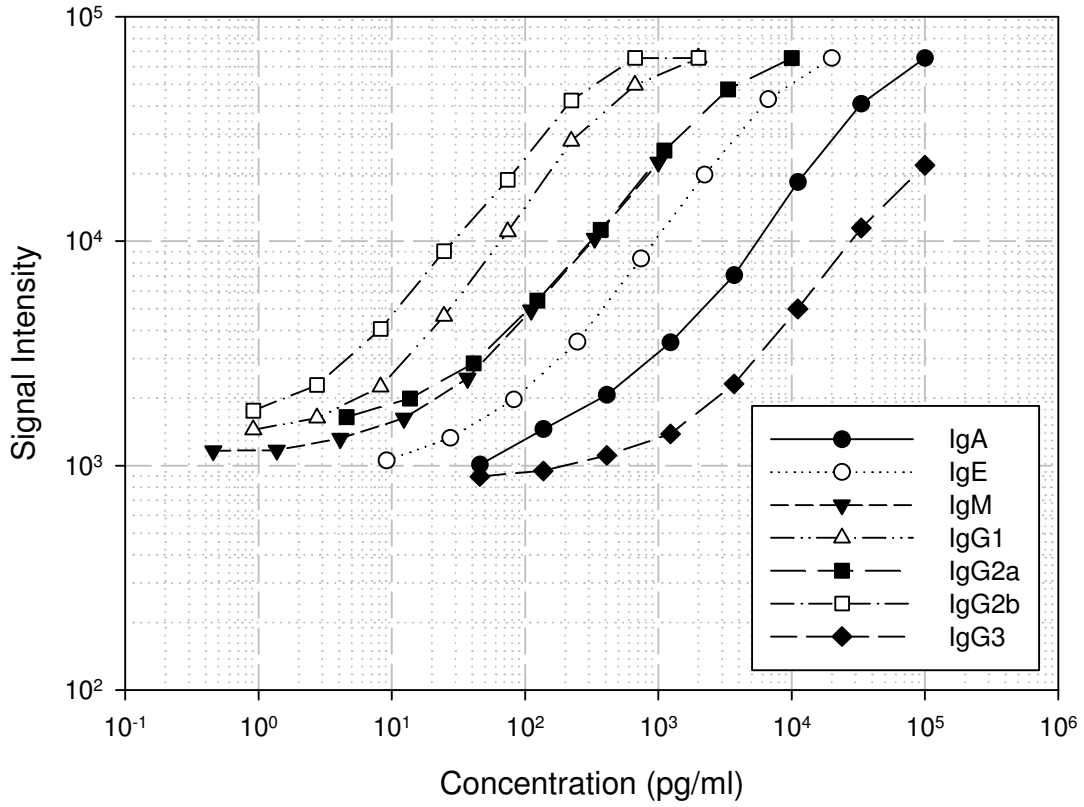
F. Data Analysis

16. Data extraction can be done with most of the microarray analysis software (GenePix, ScanArray Express, ArrayVision, or MicroVigene). For quantitative data analysis, our Quantibody® Q-Analyzer software is available. It gives visual output as well as digital values. More information can be found in section VIII.

IV. Immunoglobulin Array Map & Standard Curves

POS1	POS2
IgA	IgD
IgE	IgM
IgG1	IgG2a
IgG2b	IgG3
lambda	kappa

QAM-ISO-1 Standard Curves



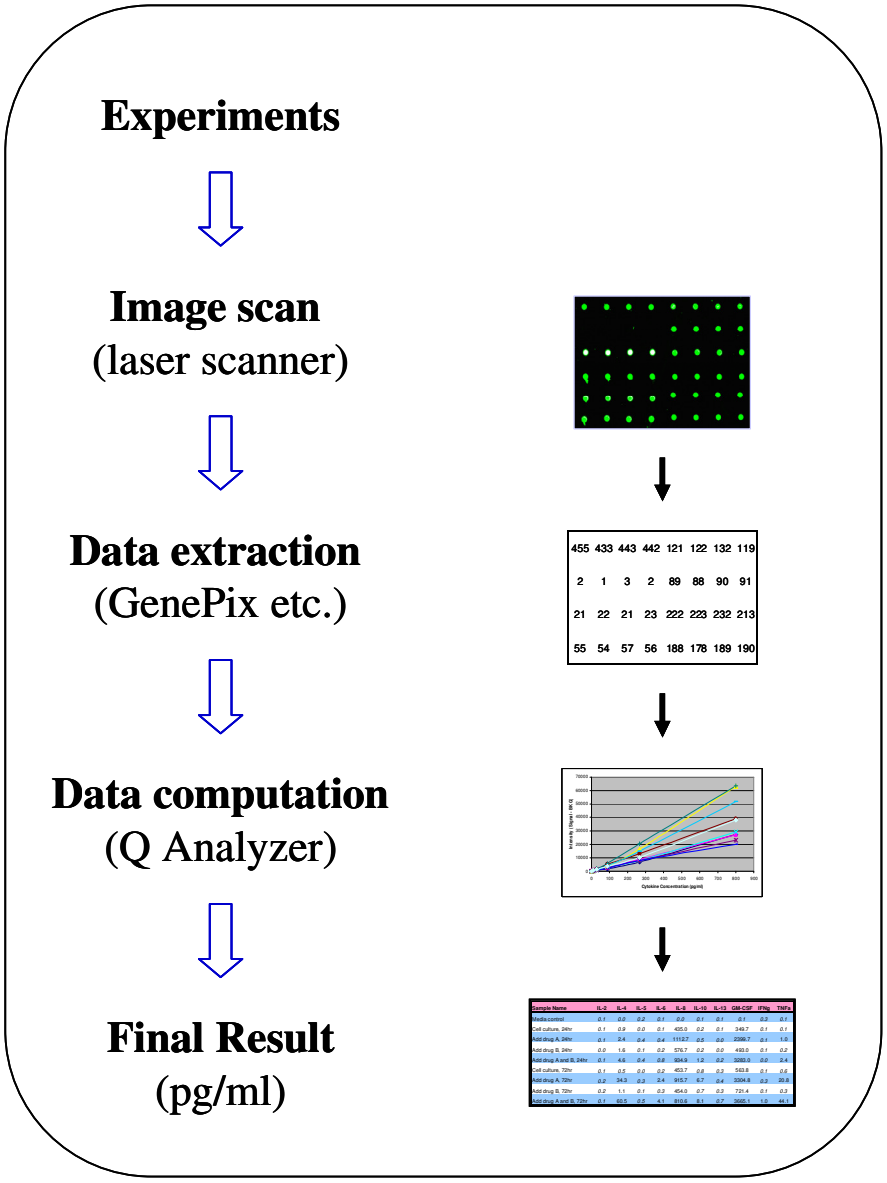
V. 8-Point Standards

After reconstitution of the lyophilized Immunoglobulin standard mix, the 8-point Immunoglobulin concentration used for generating the standard curve of a given antigen is listed below. The detection sensitivity of each protein in one experiment is user dependent. Try our array specific Quantibody Q-Analyzer to see your Limit of Detection (LOD). (Section VIII).

Serial standard concentration (pg/ml)

(pg/ml)	Control	Std7	Std6	Std5	Std4	Std3	Std2	Std1
IgA	0	137	412	1,235	3,704	11,111	33,333	100,000
IgE	0	27	82	247	741	2,222	6,667	20,000
IgM	0	1	4	12	37	111	333	1,000
IgG1	0	3	8	25	74	222	667	2,000
IgG2a	0	14	41	123	370	1,111	3,333	10,000
IgG2b	0	3	8	25	74	222	667	2,000
IgG3	0	137	412	1,235	3,704	11,111	33,333	100,000

VI. System Flow



VII. Quantibody® Q-Analyzer

Quantibody Q-Analyzer is an array specific, Excel-based program. However, it is not a simple calculation macro as it contains sophisticated data analysis.

Key features:

- *Simplicity:* Easy to operate and requires no professional training. With a simple copy and paste process, the cytokine concentration is determined.
- *Outlier Marking & Removing:* The software can automatically mark and remove the outlier spots for more accurate data analysis
- *Normalization:* The program allows for intra- and inter-slide normalization for large number of samples.
- *Two Positive Controls*: The program takes the two positive controls in each array for normalization.
- *Two Analytical Algorithms*: Users can choose either linear regression or log-log algorithms to meet their analytical needs.
- *Two Data Outputs*: standard curves and digital concentration.
- *User Intervention:* The program allows for user manual handling of those outliers and other analytical data.
- *Lower and Upper Limits Determination:* The program automatically marks out the values below or above the detection range.
- *Standard Deviation:* The program outputs the standard deviations of the quadruplicate spots for data accuracy.
- *Analytical Tips:* Q-Analyzer analysis tips are included in the program.

VIII. Troubleshooting guide

Problem	Cause	Recommendation
Weak Signal	Inadequate detection	Increase laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Short incubation time	Ensure sufficient incubation time and change sample incubation step to overnight
	Too low protein concentration in sample	Don't make too low dilution or concentrate sample
	Improper storage of kit	Store kit as suggested temperature. Don't freeze/thaw the slide.
Uneven signal	Bubble formed during incubation	Avoid bubble formation during incubation
	Arrays are not completed covered by reagent	Completely cover arrays with solution
	Reagent evaporation	Cover the incubation chamber with adhesive film during incubation
Poor standard curve	Cross-contamination from neighboring wells	Avoid overflowing wash buffer
	Comet tail formation	Air dry the slide for at least 1 hour before usage
	Inadequate standard reconstitution or Improper dilution	Reconstitute the lyophilized standard well at the room temperature before making serial dilutions. Check pipettes and ensure proper serial dilutions.
	Inadequate detection	Increase laser power that the highest standard concentration for each cytokine receives the highest possible reading yet remains unsaturated.
	Use freeze-thawed cytokine standards	Always use new cytokine standard vial for new set of experiment. Discard any leftover.
High background	Overexposure	Lower the laser power
	Dark spots	Completely remove wash buffer in each wash step.
	Insufficient wash	Increase wash time and use more wash buffer
	Dust	Work in clean environment
	Slide is allowed to dry out	Don't dry out slides during experiment.

IX. Select Quantibody Publications

1. Stechova, et al. Influence of Maternal Hyperglycaemia on Cord Blood Mononuclear Cells in Response to Diabetes-associated Autoantigens. *Scandinavian Journal of Immunology*. 2009. 70(2):149-158
2. Willingham, SB et al. NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways. *J Immunol.* 2009; 183(3):2008-15
3. El Karim et al. Neuropeptides Regulate Expression of Angiogenic Growth Factors in Human Dental Pulp Fibroblasts. *Journal of Endodontics*, 2009; 35(6): 829-833
4. Souquière S. et al. T-Cell tropism of simian T-cell leukaemia virus type 1 and cytokine profiles in relation to proviral load and immunological changes during chronic infection of naturally infected mandrills (*Mandrillus sphinx*). *J Med Primatol*. 2009; 38(4):279-89
5. Sharma, et al. Induction of multiple pro-inflammatory cytokines by respiratory viruses and reversal by standardized *Echinacea*, a potent antiviral herbal extract. *Antiviral Research*. 2009; 83(2)165-170.
6. Altamirano-Dimas, et al. *Echinacea* and anti-inflammatory cytokine responses: Results of a gene and protein array analysis. *Pharmacuetical Biology*. 2009; 47(6): 500-508.
7. Cheung, et al. Cordysinocan, a polysaccharide isolated from cultured *Cordyceps*, activates immune responses in cultured T-lymphocytes and macrophages: Signaling cascade and induction of cytokines. *Journal of Ethonopharmacology*. 2009; 124(1): 61-68.
8. Du, et al. P2-380: Identification and characterization of human autoantibodies that may be used for the treatment of prion diseases. *Alzheimer's and Dementia*. 2009; 4(4): T484-T484.
9. Van Rossum et al. Granulocytosis and thrombocytosis in renal cell carcinoma: a pro-inflammatory cytokine response originating in the tumour. *Neth J Med*. 2009; 67(5):191-4.
10. Zhai, et al. Coordinated Changes in mRNA Turnover, Translation, and RNA Processing Bodies in Bronchial Epithelial Cells following Inflammatory Stimulation. *Molecular and Cellular Biology*. 2008; 28(24): 7414-7426.
11. Gao, et al. A Chinese herbal decoction, Danggui Buxue Tang, activates extracellular signal-regulated kinase in cultured T-lymphocytes. *FEBS Letters*, 2007; 581(26): 5087-5093. (This reference validates mulitplex ELISA results for several analytes with standard ELISA test results).
12. Piganelli, et al: Autoreactive T-cell responses: new technology in pursuit of an old nemesis. (Editorial Review) *Pediatric Diabetes* 2007: 8: 249–251

X. Experiment Record Form

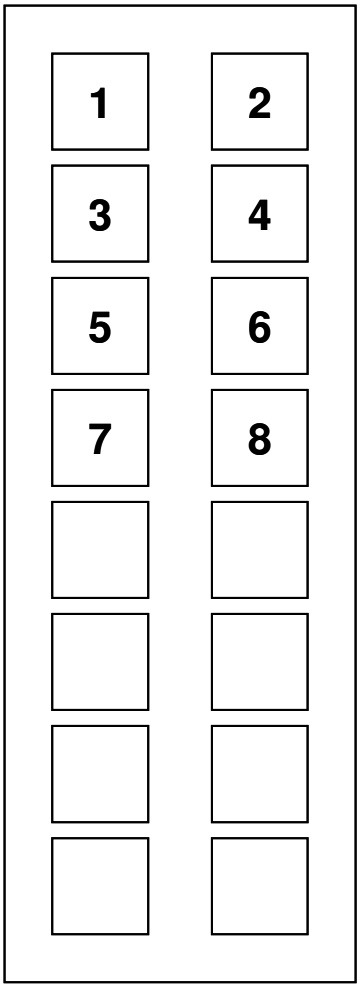
Date: _____

File Name: _____

Laser Power: _____

PMT: _____

Well No.	Sample Name	Dilution factor
1	CNTRL	
2	Std7	
3	Std6	
4	Std5	
5	Std4	
6	Std3	
7	Std2	
8	Std1	
9		
10		
11		
12		
13		
14		
15		
16		



XI. How to Choose Quantibody® Products?

Species-based selection:

- **Human**: (QAH-)
- **Mouse**: (QAM-)
- **Rat**: QAR
- **Porcine**: QAP-CYT-1
- **Non-Human Primates (NHP)**: QAN-CYT-1
- **Canine**: QAC-CYT-1
- **Feline**: QAF-CYT-1
- **Equine**: QAE-CYT-1
- **Bovine**: QAB-CYT-1
- **Rabbit:** QAL-CYT-1

Function-based selection:

- **TH1/TH2/TH17 Array:** QAH-TH-1, QAH-TH17, QAM-TH17
- **Inflammation Arrays:** QAH-INF-1, QAH-INF-2, QAH-INF-3; QAM-INF-1; QAR-INF-1
- **Angiogenesis Arrays:** QAH-ANG-1, QAH-ANG-2, QAH-ANG-3, QAH-ANG-1000
- **Chemokine Arrays:** QAH-CHE-1, QAM-CHE-1
- **MMP Array:** QAH-MMP-1
- **Immunoglobin Isotype Array:** QAH-ISO-1; AAM-ISO-G1
- **Dried Eye Disease**: QAH-DED-1
- **Periodontal Disease**: QAH-PDD-1
- **Sepsis Biomarker**: QAH-SEP-1

Cytokine Number-based selection:

- **440 cytokines:** QAH-CAA-440
- **400 cytokines:** QAH-CAA-9000
- **360 cytokines:** QAH-CAA-8000
- **320 cytokines:** QAH-CAA-7000
- **280 cytokines:** QAH-CAA-6000
- **240 cytokines:** QAH-CAA-5000
- **200 cytokines:** QAH-CAA-4000; QAM-CAA-4000
- **160 cytokines:** QAH-CAA-3000, QAM-CAA-3000
- **120 cytokines:** QAH-CAA-2000; QAM-CAA-2000
- **80 cytokines:** QAH-CAA-1000; QAM-CAA-1000
- **60 cytokines:** QAH-ANG-1000; QAM-CYT-Q2000
- **40 cytokines:** QAH-INF-3, QAH-CHE-1, QAH-GF-1, QAH-REC-1, QAH-CYT-4,
- **30 cytokines:** QAH-ANG-2, QAH-ANG-3, QAM-INT-1000, QAR-CYT-3, QAM-CHE-1
- **20 cytokines:** QAH-CYT-1, QAH-CYT-2, QAM-CYT-1, QAM-CYT-2, QAM-CYT-3
- **10 cytokines:** QAH-TH-1, QAH-INF-1, QAH-INF-2, QAH-ANG-1, QAH-MMP-1
- **less than 10 cytokines:** QAH-ISO-1, QAH-ADI-2, QAP-CYT-1, QAM-ISO-G1

Purpose-based selection --- Custom Arrays

- Choose from over 1000 cytokine pool; Any kind; Any number
- Order slide only or full service in house
- Desired marker not in our pool? No problem! For certain developmental fee, we may be able to add the marker to your panel if the paired antibodies are available on the market.

Note:

Quantibody® is the trademark of RayBiotech, Inc.

Cytokine protein arrays are RayBiotech patent-pending technology.

This product is intended for research only and is not to be used for clinical diagnosis. Our produces may not be resold, modified for resale, or used to manufacture commercial products without written approval by RayBiotech, Inc.

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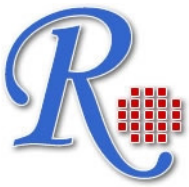
This product is for research use only.



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